# TITLE: PURINE NUCLEOSIDES AS ANTI-APOPTOTIC AGENTS

## FIELD OF THE INVENTION

The invention relates to methods and compositions for preventing apoptosis.

## BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death, is a gene-mediated mechanism associated with normal physiology and biological processes like proliferation and differentiation. Apoptosis plays an important role in normal neuronal development and in acute chronic pathological processes such as trauma, stroke, Huntington's chorea, prion diseases such as Creudzfeldt-Jacob disease, scrapie and bovine spongiform encephalopathy, and Alzheimer's disease (McConkey and Orrenius, 1994). How particular types of cells are targeted or protected is accomplished by activating genes that encode products such as caspases (cysteine-aspartate proteases) to commit cell suicide or proteins of the Bcl-2 family of proteins to protect them.

Apoptosis can be divided essentially into three phases; initiation, effector and a final outcome of cell death. The initiation phase may be activated by either an external (TNF receptor, Fas ligand) or an internal signal from the cell (oxidative stress or excess $Ca^{2+}$ release) to activate specific signal transduction pathways. After a signal has been initiated, "check points" mediate the effector stage such as, gene products of the Bcl-2 related family of proteins and/ or cysteine aspartate proteases. It is the relative abundance of pro-apoptotic and/or the anti-apoptotic signals that will determine if a cell will be committed to undergo apoptosis. The final outcome of apoptosis is fragmented apoptotic bodies, which are eliminated by phagocytic cells without an inflammatory response. It is important to recognize that apoptosis is not a single process, but rather several process. Thus an intervention that prevents apoptosis in one condition may have no effect on apoptosis in other conditions.

Purines, in the nervous system (central and peripheral) can act as neurotransmitters and mediate trophic effects. These effects may lead to changes in cell metabolism, proliferation, and/ or release of certain growth

factors. Purine nucleosides (adenosine, guanosine, inosine) are known to have many neuroprotective and neurorestorative effects in the peripheral and central nervous system.

## SUMMARY OF THE INVENTION

The present inventors have demonstrated that purines nucleosides such as guanosine and inosine are capable of exerting an anti-apoptotic signal in cells of the central nervous system. The inventors have also shown that guanosine acts through a PI 3-K/ Akt (protein kinase B) pathway that is capable of protecting cells from further undergoing the process of apoptosis.

Accordingly, the present invention provides a method for preventing apoptosis in a cell of the nervous system comprising administering an effective amount of a purine nucleoside to a cell or animal in need thereof.

The present invention also includes a pharmaceutical composition for use in preventing apoptosis comprising an effective amount of a purine nucleoside in admixture with a suitable diluent or carrier.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a bar graph showing the effect of guanosine on staurosporine-induced apoptosis and intracellular pathways.

Figures 2A and B show guanosine-mediated phosphorylation of Akt (protein kinase B) in cultured rat astrocytes as evaluated by Western blot analysis.

Figure 3A-C shows the effect of guanosine on phosphorylation of Akt/PKB in cultured rat astrocytes.

Figure 4 demonstrates that guanosine promotes phosphorylation of glycogen synthase kinase-3β (GSK-3β).

Figure 5 demonstrates that guanosine induces the expression of Bcl-2 mRNA and protein in rat cultured astrocytes.

Figure 6 is a bar graph showing the effect of propentofylline, pertussis toxin, and antagonists of adenine-base purines on the protective effect of guanosine against staurosporine-induced apoptosis in cultured astrocytes.

## DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present inventors have demonstrated that the purine nucleosides guanosine and inosine are capable of preventing apoptosis in cells of the nervous system, such as neurons and astrocytes. The inventors have shown that guanosine can protect against apoptotic cell death caused by a number of known inducers or stimulators of apoptosis. In one example, the inventors have shown that guanosine can protect astrocytes from apoptosis induced by staurosporine which is a potent inducer of apoptosis. In another example, the inventors have shown that guanosine can protect neuronal cells from apoptosis induced by β-amyloid protein which is known to induce apoptosis of neurons in Alzheimer's disease. These results demonstrate the applicability of the present invention to treating Alzheimer's disease. In a further example, the inventors have demonstrated that guanosine protected astrocytes and neuroblastoma cells from apoptosis induced by combined hypoxia-hypoglycemia using combined oxygen-glucose deprivation (CGOD). As CGOD is an *in vitro* model of cerebral ischemia, the results demonstrate the applicability of the method of the invention in treating conditions involving cerebral ischemia such as stroke or cerebral hypoxia due to circulatory insufficiency.

Accordingly, the present invention provides a method of preventing apoptosis in a cell of the nervous system comprising administering an effective amount of a purine nucleoside to a cell or animal in need thereof. The present invention also provides a use of an effective amount of a purine nucleoside for the manufacture of a medicament for preventing apoptosis in a cell of the central nervous system.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result (e.g. preventing apoptosis).

The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

The term "a cell" as used herein includes a single cell as well as a plurality or population of cells. Administering a purine nucleoside to a cell includes both *in vitro* and *in vivo* administrations.

The term "a cell of the nervous system" includes cells from both the peripheral and central nervous system. Preferably the cell is from the central nervous system, most preferably the cell is an astrocyte or a neuron.

The term "preventing apoptosis" means that the level of apoptosis in the presence of the purine nucleoside is decreased or reduced as compared to the level of apoptosis observed in the absence of the purine nucleoside. Apoptosis can be measured using a variety of known techniques, including measuring the activity of caspases, assessing DNA fragmentation (e.g. using the Tunel assay described in Example 1) or by assessing cell viability (e.g. using staining method described in Example 1).

The inventors have shown that guanosine exerts its anti-apoptotic effect through a PI 3-kinase/Akt/protein kinase β pathway. In particular, they have shown that the anti-apoptotic effect of guanosine is mediated by inactivation of glycogen synthase kinase-3β (GSK-3β), a downstream target of the PI3K/Akt/PKB pathway. Accordingly, the present invention provides a method of inhibiting glycogen synthase kinase-3β comprising administering an effective amount of a purine nucleoside to a cell or animal in need thereof. The present invention also provides a use of an effective amount of a purine nuecleoside for the manufacture of a medicament for inhibiting the activity of glycogen synthase kinase-3β in a cell of the central nervous system.

The inventors have also shown that guanosine induces Bcl-2 which is a known anti-apoptotic protein. Accordingly, the present invention provides a method of inducing the expression and/or activity of Bcl-2 in a cell of the nervous system comprising administering an effective amount of a purine

nucleoside to a cell or animal in need thereof. The present invention also provides a use of an effective amount of a purine nucleoside for the manufacture of a medicament for inducing the expression and/or activity of Bcl-2 in a cell of the central nervous system.

The purine nucleoside used in the above methods is preferably selected from guanosine, inosine and analogs thereof. Examples of analogs are provided below.

Derivatives by modifying the 2-amino-$NH_2$ group of guanosine:

| | |
|---|---|
| 2 – methyl - | 2-$CH_3$- |
| 2 – ethyl - | 2 – $C_2H_5$ – |
| 2 – ethylamino - | 2- $CH_3CH_2NH$ – |
| N,N – dimethyl - | 2 – $(CH_3)_2N$ – |
| 2 – methylamino - | 2 – $CH_3NH$ – |
| 2 – ethylamino - | 2 – $CH_3CH_2NH$ – |
| $N_2$ – benzoyl - | 2 – $C_6H_5C$ – NH – |
| phenyl – amino - | 2 – $C_6H_5NH$ – |

Substituting – phenyl – amino –

Derivatives by modifying the 6 – keto – C=O group of guanosine or inosine:

| | |
|---|---|
| 6 – thio - | 6 – SH – |
| 6 – amino - | 6 – $NH_2$ – |
| 6 – chloro - | 6 – Cl – |
| 6 - methoxy - | 6 – $OCH_3$ – |
| 6 - cyclopentyl - | 6 – $C_5H_9$ – |
| 6 – cyclohexyl - | 6 – $C_6H_{11}$ – |

Derivatives by substituting the imidazole ring of the purines:

| | |
|---|---|
| N7 – methyl - | 7 – $CH_3$ – |
| C8 – hydroxy - | 8 – OH – |
| C8 – bromo - | 8 – Br – |

Derivatives by modifying the ribose ring of purine nucleosides:

2' – deoxy - ribose

2',3' – dideoxy – ribose

5' – carbamido – derivatives

Changing the ribose to other sugars – eg: arabinose

Changing the ribose to carbocyclic analogues – eg: cyclopentane

Whether or not a particular analog of guanosine or inosine is useful in the methods of the invention can be tested using assays known in the art including the assays described herein. For example, the analog can be tested for its ability to prevent apoptosis using the assays described in the Examples. The analog can also be tested for its ability to inhibit GSK-3β or to induce Bcl-2 using the assays described in the Examples.

The method of the invention can be used to treat any disease or condition of the nervous system wherein it is desirable to prevent apoptosis including, but not limited to, neurodegenerative diseases (including Alzheimer's disease, Huntington's chorea, Parkinson's disease, Bell's Palsy, Pick's disease, multiple sclerosis and amyotrophic lateral sclerosis), stroke, head injuries, and spinal cord and other nerve crush injuries. The disease may also be a prion-induced disease such as Creudzfeldt-Jacob disease, scrapie, and bovine spongiform encephalopathy.

The present invention also includes pharmaceutical compositions containing purine nucleosides to prevent apoptosis in a cell of the central nervous system. Accordingly, in one embodiment, the present invention provides a pharmaceutical composition for use in preventing apoptosis comprising an effective amount of a purine nucleoside in admixture with a suitable diluent or carrier. The purine nucleoside is preferably guanosine, inosine or analogs thereof.

The pharmaceutical compositions can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as other agents that can prevent the inhibition of apoptosis or that are used in treating inflammatory conditions or sepsis.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The following non-limiting examples are illustrative of the present invention:

**EXAMPLE**

**Example 1**

**MATERIALS AND METHODS**

**Cells**

***SK-N-SH culture***

The SK-N-SH human neuroblastoma cells were purchased from ATCC and grown in Minimum Essential Medium (MEM) (Gibco, Burlington, ON) at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. MEM was supplemented with non-essential amino acids (0.1 mM), sodium pyruvate (0.1 mM), penicillin / streptomycin 100 U/ ml, and 10% fetal bovine serum (FBS) (Gibco, Burlington, ON). Cells were treated with 236 nM herbimycin A (Sigma, Mississauga, ON) and 10 µM retinoic acid (Sigma, Mississauga, ON) in order to differentiate the cells into a neuronal type (Preis et al., 1988). For

individual experiments, SK-N-SH cells were harvested by washing cells one time with warmed 0.1 M phosphate buffered saline, and then treating the cells with 1 ml of trypsin-EDTA (0.05%, 0.53 nM) (Gibco BRL, Burlington, ON). Cells were centrifuged at 1000 rpm for 5 minutes. Cells were re-suspended in supplemented MEM and counted in the presence of Trypan blue (0.04%) using a hemocytometer to exclude non-viable cells. Cells were seeded at a concentration of 2.5 x $10^4$ cells/well in a 24 well Falcon tissue culture plate and grown for 2 days. Cells were serum starved for 24 hours using supplemented MEM plus 0.5% FBS.

***Astrocyte culture***

Primary cultures of rat astrocytes were prepared from cerebral cortex of 18-19 day-old-fetuses of Sprague Dawley rats as previously described (Di Iorio et al., 2002). Dissociated cortical cells were grown on poly-D-lysine-coated (10 µg/ml) T75 flasks using Dulbecco's Modified Eagle Medium (DMEM) culture medium containing 20% horse serum (HS) and 1% penicillin / streptomycin. Cultures were maintained in standard conditions, 37°C; 100% humidity; 95% air/5% $CO_2$. Initially atrocytes were treated with a lysomotrophic agent, L-Leucine methyl ester (5mM) (Sigma, Oakville, ON), to selectively destroy contaminating microglia (Giuliam and Baker, 1986). After which they were maintained in high glucose DMEM containing 5% HS and 1% penicillin / streptomycin with medium replacement every 3-4 days. After 12-14 days in vitro (DIV) the cells were agitated vigorously for 4 hours on an orbital shaker at 100 rpm to minimize microglial contamination (Giulian and Baker, 1986). Immunostaining for glial fibrillary acidic protein (Reinhart et al., 1990) showed that more than 97% of cultured cells were astrocytes, which had the typical appearance of type-1 astrocytes upon microscope examination. Astrocytes were detached from the culture flasks by treatment (5-10 min, 37°C) with 0.25% Trypsin/0.04% EDTA (GibcoBRL). Cells were counted in the presence of 0.04% Trypan Blue Stain (GibcoBRL) to exclude non-viable cells; this showed 97% viability. Astrocytes were resuspended in DMEM supplemented with 20% HS and 1% penicillin / streptomycin and seeded as follows: for the evaluation of apoptosis, cells were replated onto poly-D-lysine-

coated round glass coverslips at cell concentration of $1x10^5$ cells/coverslip; for Western blot and RT-PCR analyses astrocytes were replated onto poly-D-lysine-coated 100 mm dishes, respectively, at a cell concentration of $2x10^5$ cells/dish. On the 2nd day after replating, media was replaced with DMEM supplemented with 5% HS and 1% penicillin / streptomycin and bioassays were usually performed on the cultures 4 days after replating.

**Treatment of cell cultures with various drugs**

Astrocytes and SK-N-SH cells were treated with a variety of purines, at different concentrations as follows: guanosine 10-300 µM or inosine 100 µM. In experiments, which evaluated apoptosis, astrocytes and/ or SK-N-SH cells were treated with 100 nM or 10 nM staurosporine, a potent inducer of apoptosis. Staurosporine is a potent inducer of apoptosis in neuronal and non-neuronal cells (Ahlemeyer et al., 2000; Koh et al., 1995; Mangoura and Dawson, 1998; Weisner and Dawson, 1996; Krohn et al., 1998, 1999). Cells were treated with amyloid β-protein (25-35) at a final concentration of 5 µM and 10 µM. In experiments where cells were pre-treated with either purine nucleoside, the nucleoside was added one hour prior to the addition of either staurosporine or amyloid β-protein. In experiments to determine the effect of intercellular transduction pathways, astrocytes were treated with [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole,FHPI] (SB202190), an inhibitor of the p38 mitogen-activated protein kinase pathway or [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002) (Calbiochem), an inhibitor of phosphatidyl inositol 3- kinase or with adenosine receptor antagonists, DPCPX (100nM) or alloxazine (10 µM) or P2 purine receptor antagonists, suramin (10 µM).

Certain experimental agents were dissolved in dimethylsulfoxide (DMSO) or sodium hydroxide (NaOH, 1N), all experiments were performed using solubility controls at a final concentration of 0.01% DMSO or 0.01% NaOH. All purines were purchased from Sigma unless otherwise stated.

**Determination of apoptosis**

**a. Tunel assay**

DNA fragmentation was evaluated using the In Situ Cell Death Detection Kit: TUNEL assay [TdT(terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling] (Boehringer Mannheim) and performed according to the manufacturers instructions. Briefly, at the end of the treatments adherent astrocytes were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with phosphate buffered saline and incubated in permeabilization solution (0.1% triton, 0.1% sodium citrate) for 20 minutes on ice (4°C) and assayed by TUNEL. Positive controls were included by incubating the fixed and permeabilized cells with DNase I (10 µg/ml) for 10 minutes at room temperature to induce DNA strand breaks. Finally the cells were examined by fluorescence microscopy.

**b. Staining with acridine orange / ethidium bromide**

SK-N-SH cells were stained for cell viability or apoptosis using acridine orange (AO) (3 µg/ml) or ethidium bromide (EB) (10 µg/ml) in 0.1 M phosphate buffer saline (PBS) solution. After treatment exposure, cells were rinsed twice with 0.1 M PBS and treated with AO/EB solution. Cells were visualized under fluorescence (485 nm). Live cells stained green and dead cells fluoresced orange/ red. Four individual fields were counted / well / treatment.

**Western blot analyses**

Phosphorylation of Akt / protein kinase B was evaluated by Western blot analysis. After 72 hours of re-plating astrocytes were serum starved in high glucose DMEM for 24 hours. Astrocytes were exposed to increasing concentrations of guanosine (30, 100 and 300µM) for 10 minutes at 37°C. In experiments using the inhibitor of PI 3-kinase, LY294002 (30 µM) was added to the culture medium 30 minutes prior to the addition of guanosine. At the end of the treatments, astrocytes were washed twice with ice-cold phosphate buffered saline (PBS) and harvested at 4°C in 25 mM Tris buffer pH 7.4 containing: 150 mM NaCl, 100 µM sodium orthovanadate, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1% NP40, 10% glycerol, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin. Cells were disrupted by sonication and centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots (20 µl) were removed from the supernatants for the

determination of protein concentration. Samples were diluted in SDS-bromophenol blue buffer and boiled for 5 min before loading onto 12.5% SDS polyacrilamide gel, using 10 µg of total protein per lane. After separation, proteins were transferred to immuno-Blot PVDF membranes (Bio-Rad Laboratories). Membranes were incubated with an antibody against phosphorylated Akt [1:1000, rabbit polyclonal phospho-Akt (Ser473) antibody (New England Biolabs)] overnight at 4°C. Blots were then incubated for 1 hour with the secondary antibody (peroxidase-coupled anti-rabbit, 1:2000, Amersham). Immunostaining was revealed by the enhanced ECL western blotting analysis system (Amersham). Densitometric analysis was performed for quantification of the immunoblots. Assay of Caspase-3 Activity

The activity of caspase-3 was determined using a colorimetric protease assay kit (MBL, Nagoya, Japan). Cell lysates were prepared from $2x10^6$ rat cultured astrocytes after various treatments. Aliquots of the lysates were diluted in 50µl of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation at 10,000 x g for 1 min, the supernatants were stored on ice. A proteolytic reaction was carried out in a reaction buffer containing 50µg of cytosolic protein extract and 200µM of N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA). The reaction mixture was incubated at 37°C for 2 h and the formation of p-nitroanilide (pNA) was measured at 405 nm using a microtiter plate reader (Spectracount™, Packard Canberra, IL, USA). The activity of caspase-3, was calculated from the slope of absorbance over time, and is expressed as pmol of substrate cleaved/min/µg of cytosolic protein.

**Identification of Bcl-2 mRNA by Northern Blot Analysis**

Cultured rat astrocytes were pre-treated with guanosine (300 µM) for 4 hours, and total RNA was isolated from untreated and guanosine-treated cells using the TRIzol reagent (Life Technologies BRL, Milan, Italy) and quantified by a scanning spectrophotometer. Twenty micrograms of total RNA was electrophoresed on formaldehyde denaturing 1 % agarose gel and viewed under ultraviolet light (UV) to verify loading and the presence of intact ribosomal bands. The RNA was then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, UK) and fixed

by UV crosslinking (UV Stratalinker 1800, Stratagene). Bcl-2 cDNA probe was amplified by the polymerase chain reaction (PCR). Bcl-2 primers were designed based on a previously published rat cDNA sequences (Genebank accession number, RNBCL2A): Bcl-2 primer sense 5'-CGGGACGCGAAGTGCTAT-3', Bcl-2 primer antisense 5'-CCCAGCCTCCGTTATCCTGGA-3'. Bcl-2 insert was purified and radiolabeled by the method of random priming (Megaprime DNA labeling system, Amersham Pharmacia Biotech) using $^{32}$P-labeled alpha-dCTP. Filters were prehybridized in a mixture containing 50% formamide, 1% sodium dodecyl sulphate (SDS), 5 x tri-sodium citrate, sodium chloride (SSC), 5 x Denhardt's solution, and 100 mg/ml denatured salmon sperm. Hybridization was performed under the same conditions with the addition of labeled probe at 42°C overnight. Membranes were washed using 0.1% SDS/2 x SSC at room temperature and then twice in 0.2% SDS/1 x SSC at 50°C before being exposed to Kodak Biomax MS film (Sigma). Subsequent to hybridization with Bcl-2 cDNA probe, filters were hybridized with a probe for GAPDH to allow correction for the recovered RNA in each sample. For the purpose of quantification autoradiograms were scanned using laser densitometry. Bcl-2 mRNA signals were normalized against GAPDH content by determining the ratio of their respective optical densities.

**RT-PCR**

Effect of guanosine on the expression of Bcl-2 mRNA in rat cortical astrocytes was also determined by semi-quantitative RT-PCR. Total RNA was isolated from cultured rat astrocytes, untreated and treated with 150 µM guanosine for 4 hours, and 2 µg were reverse-transcribed to cDNA. Standard curves were generated using serial dilutions of the RT reactions as templates for PCR with each primer set to determine the linear range of the assay. Products from the original RT reaction volumes were appropriately diluted into the final PCR volumes to ensure nonsaturation of the PCR amplification reactions.

**RESULTS**

***The anti-apoptotic effect of guanosine mediated by a Gi protein-coupled putative receptor for this nucleoside***

The inventors have recently reported that specific binding sites exist for [$^3$H]-guanosine in rat brain membranes (Traversa et al., 2002a), and these putative receptor sites are also expressed in cultured rat astrocytes (Traversa et al., 2002b). In the latter cells, as well as in whole brain membranes, guanosine binding is sensitive to treatment with pertussis-toxin (PTX), suggesting that the putative guanosine receptor is coupled to $G_i$ proteins (Traversa et al., 2000; Traversa et al., 2002b). The inventors therefore investigated whether the anti-apoptotic effect of guanosine was mediated by these plasma membrane localized guanosine receptors. Astrocytes were exposed to 100nM staurosporine for 3 hours, and 300μM guanosine or vehicle (growth medium = control) was added 1 hour prior to the addition of staurosporine, and was present during the 3 hours of staurosporine treatment (total exposure time 4 hours). Cells were pre-treated with propentofylline (PPF, 100 μM), or the combined antagonists of adenine-base purines (ABP) (100 nM DPCPX + 30 μM DMPX + 10 μM suramin) for 30 min prior to the addition of guanosine. Pre-treatment with pertussis toxin (PTX, 200 ng/ml) was performed for 16 hours. The extent of apoptosis was analyzed by the oligonucleosome ELISA assay 24 hours after the start of the experiment. Results are expressed as a percentage of apoptotic cells of the total number of cultured cells. Data are the mean ± S.E.M. of 5 independent experiments. Significant difference vs. control: *$p<0.05$; **$p<0.02$, (Student's t test).

The results are shown in Figure 6 and show that PTX abolished the anti-apoptotic effect of guanosine. The number of apoptotic cells in PTX-treated astrocytes was not significantly different from that elicited by staurosporine alone. These results indicate that the protection by guanosine is mediated by a $G_i$-protein coupled plasma membrane binding site for guanosine, and this is most likely the putative guanosine receptor.

***Guanosine-mediated phosphorylation of Akt/PkB in astrocytes***

Guanosine-mediated phosphorylation of Akt (protein kinase B) in cultured rat astrocytes was evaluated by Western blot analysis. The results

are shown in Figure 2. After 72 hours of re-plating, astrocytes were serum starved in high glucose for an additional 24 hours. Astrocytes were treated in panel A with control or with increasing concentrations of guanosine for 10 min. In panel B, astrocytes were pre-treated for 30 min with either the selective A1 adenosine receptor antagonist (DPCPX 100 nM) plus the selective A2B receptor antagonist (alloxazine 10 μM) plus the non-selective P2 purine receptor antagonist (suramin 10 μM), or with the PI-3 kinase inhibitor LY294002 (30 μM) prior to the addition of 300 μM guanosine. The antagonists were maintained also during the stimulation period (10 min) with guanosine. Cell lysates (10 μg) were resolved by SDS-PAGE and transferred to PVDF membranes before being probed with antisera specific for phosphorylated PKB ($Ser^{473}$). The immunoblots are representative of 4 independent experiments. Blots were analysed by densitometric analyses and results are reported in the panels under each blot. In the panels A and B, the values are given as means ± S.E.M. of four experiments.

*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: significant difference versus control (Student's two-tailed t-test).

Figure 3 also shows guanosine-mediated phosphorylation of Akt/PKB in cultured rat astrocytes.

a) Effect of increasing concentrations of guanosine (Guo) on Akt/PKB phosphorylation.

Astrocytes were grown in medium containing 2% FBS for 24 hours, then were exposed to varying concentrations of guanosine (30, 100 or 300 μM) for 10 minutes. At the end of the indicated treatment times astrocytes were washed twice with ice-cold phosphate buffered saline (PBS) and harvested at 4°C. The expression of phosphorylated Akt/PKB was determined by Western immounoblot analysis as described in the Methods section. Immunoblots were quantitated by densitometric analysis, and reported in the panels below each blot. Data presented are representative of 4 independent experiments, with similar results. Maximal phosphorylation of Akt/PKB is detected following exposure of astrocyte cultures to 300 μM guanosine.

b) Time course of guanosine-stimulated Akt/PKB phosphorylation.

Experiments were performed as described for Figure 2, and astrocytes were exposed to 300µM guanosine (Guo) for varying times, and the expression of phosphorylated Akt/PKB was determined as in Figure 2. Maximal phosphorylation of Akt/PKB is detected after 5 minutes of guanosine exposure of astrocytes.

c) Effect of inhibitors of PI-3 kinase ( LY 294002) and the combined antagonists of adenine-base purines (ABP) (DPCPX + DMPX + suramin) on the guanosine (Guo)-stimulated Akt/PKB phosphorylation.

Experiments were performed as described for Figure 2. Astrocytes were pre-treated for 30 minutes prior to the addition of guanosine with either the inhibitor of PI-3 kinase (30 µM LY 294002), or the combined antagonists of adenine-base purines (ABP) (100 nM DPCPX + 30 µM DMPX + 10 µM suramin), and the expression of phosphorylated Akt/PKB was determined as in Figure 2. Treatment of astrocytes with the PI-3 kinase inhibitor reduced guanosine-stimulated Akt/PKB phosphorylation to values below basal levels. Exposure of astrocytes to the combined antagonists of adenine-base purines (ABP) had no significant effect on guanosine-stimulated Akt/PKB phosphorylation.

***Effect of guanosine on pro-apoptotic and cell survival pathways***

In several cell types, staurosporine-induced apoptosis has been associated with the activation of multiple intracellular effectors and pathways, such as caspases, the efflux of cytochrome c from mitochondria (Deshmukh and Johnson, 2000; Belmokhtar et al., 2001), or the p38 MAP kinase pathway (Mirkes et al., 2000). The inventors therefore pre-treated astrocytes with the selective inhibitors of p38 MAP kinase (SB 202190, 1 µM), or caspases (z-VAD-fmk, 200 µM), and evaluated the effect of these agents on staurosporine-induced apoptosis. Administration of SB 202190, or z-VAD-fmk alone or in combination, reduced, but did not abolish the pro-apoptotic activity of staurosporine (by 68.2 ± 4.2% or 75.4 ± 3.9%, respectively, and by 86.2 ± 5.3% when administered in combination) (Table 4). These data suggest, that

the pro-apoptotic mechanisms of staurosporine in astrocytes involve p38 MAP kinase and caspase activation.

Pre-treatment of astrocytes with guanosine for 1 hour, prior to the addition of staurosporine reduced the number of apoptotic cells from 53.6 2 ± 4.9 % to 23.0 2 ± 3.1 %; $p < 0.001$. When cells were pre-treated for 15 minutes with either SB 202190 or z-VAD-fmk alone or in combination, prior to the addition of guanosine, the number of apoptotic cells was again reduced (Table 4). These results therefore suggest that the anti-apoptotic effect of guanosine may be mediated, at least in part, by the p38 MAP kinase pathway and the caspase system.

To test whether other intracellular survival pathways are also involved in the anti-apoptotic activity of guanosine, the inventors pre-treated astrocytes with 30 μM LY 294002, a selective inhibitor of the PI 3-kinase prior to the administration of guanosine. The addition of LY 294002 had no significant effect on the apoptosis-promoting activity of staurosporine, whereas it completely abolished the anti-apoptotic effect of guanosine (Table 4). These data indicate that one of the main pathways activated by guanosine to reverse staurosporine-induced apoptosis of astrocytes includes the intracellular enzyme, PI 3-kinase.

Figure 1 is a bar graph showing the effect of guanosine on staurosporine-induced apoptosis and intracellular pathways. Effect of selected inhibitors of intracellular pathways on the percent of apoptotic astrocytes exposed to staurosporine in the absence or presence of guanosine. Astrocytes were exposed to guanosine (300 μM) for 1 hour before being exposed to staurosporine (100 nM) for 3 hours. At the end of this time period, the media was removed and replaced with DMEM without the pharmaceutical agents, and maintained for the entire duration of the experiment. When present, astrocytes were pre-treated for 30 minutes before the addition of guanosine with the following inhibitors: [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002), an inhibitor of PI 3-kinase or [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI] (SB202190), an inhibitor of the p38 pathway. Astrocyte apoptosis was

analyzed at the 48h-time point by TUNEL assay as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the total cell number. Values are means ± SEM of at least 4 independent experiments. Statistical significance was evaluated by Student's *t*-test;

** $P < 0.005$ compared to staurosporine treatment alone.

* $P < 0.05$ compared to staurosporine treatment alone.

***Guanosine inhibits glycogen synthase kinase-3b (GSK-3β)***

One of the physiological substrates of Akt/PKB in the cell survival pathway is GSK-3β (Papp and Cooper, 1998; Hetman et al., 2000). The inventors therefore evaluated whether treatment of cultured astrocytes with guanosine had any effect on this intracellular target. Astrocytes, were grown in culture medium containing 2% FBS for 24 hours, then were treated with 300μM guanosine (Guo) for the indicated time periods. Cell lysates (60 μg of protein per lane) were analysed by Western immunoblots using a specific antibody against phospho- glycogen sythases kinase-3 (GSK-3β, Ser9), the isoform of GSK-3β phosphorylated by the activation of the PI-3K/AKT pathway. Immunoblots were quantified by densitometry as described in the Methods section. Data presented are representative of at least three independent experiments with similar results. Maximal phosphorylation of GSK-3β is detected after 5 minutes of guanosine exposure of astrocytes.

Exposure of cultured astrocytes to 300 mM guanosine for 30 min increased phosphorylation of GSK-3b at serine 9, in a time dependent manner, inhibiting the pro-apoptotic activity of this enzyme. Phosphorylation of GSK-3β was rapid, and was maximal by 5 minutes following guanosine addition, and declined to near baseline values after 30 minutes of guanosine exposure (Figure 4). These results indicate that in cultured astrocytes the anti-apoptotic effect of guanosine is mediated by inactivation of GSK-3β, a downstream target of the PI3K/Akt/ PKB pathway.

***Guanosine induces Bcl-2 mRNA and protein expression***

Another important downstream target of the PI3K/Akt/PKB cell survival pathway is the Bcl-2 protein (Hengartner, 2000), which plays an important

anti-apoptotic role. In the present study the inventors determined the expression of Bcl-2 mRNA and Bcl-2 protein in astrocytes, cultured in 2 % FBS containing medium and in cells following guanosine administration. Cultured rat astrocytes were pre-treated with guanosine (300 μM) for 4 hours, and total RNA was isolated from untreated and guanosine-treated cells as described in the Methods section. RNA was transferred to membranes and hybridized with $P^{32}$ -labelled Bcl-2 cDNA, synthesized and amplified as described in the Methods. Bcl-2 mRNA was analysed by gel eletrophoresis on 1 % agarose gel, and normalized against GAPDH. The expression of Bcl-2 protein was detected by Western immunoblot analysis, and normalized against β-actin and the results are shown in Figure 5. The top right panel presents a representative Northern blot of Bcl-2 mRNA expression and the right panel a representative Western immunoblot of Bcl-2 protein concentration. The lower panel presents the results of densitometric analyses of the Northern blots of Bcl-2mRNA, normalized to GAPDH, and the Western blots of Bcl-2 protein, normalized to β-actin at 2, 4 and 6 hours after guanosine treatment. Data are means ± S.E.M. of three independent experiments.

It was found that guanosine administration induced the expression of Bcl-2 mRNA 4 hours after exposure to this nucleoside, and it increased Bcl-2 protein expression 6 hours after guanosine exposure (Figure 5). These results confirm that in cultured astrocytes one of the intracellular targets mediating the protective effect of guanosine is the anti-apoptotic protein, Bcl-2.

**Guanosine protects agaist apoptosis induced in astrocytes and neuron-like cells by exposure to deprivation of glucose and oxygen, a model of cerebral ischemia.**

Guanosine protected against apoptotic death induced by the exposure of astrocytes and neuroblastoma cells to combined hypoxia-hypoglycemia (combined oxygen-glucose deprivation) (CGOD) for 1 hour. CGOD is an in vitro model of cerebral ischemia, as in stroke or cerebral hypoxia due to circulatory insufficiency.

This kind of insult provokes a reduction of about 20 % in the number of total cells which remained attached to the dishes or glass coveslips after washing cells for further assays (fixation and staining by DAPI or MTS test), performed 24 h after cell exposure to 1 h CGOD. In astrocytes not exposed to CGOD (basal) the percentage of apoptotic cell was about 11%. This number increased to 27% when cells were submitted to 1 h CGOD. Guanosine (300 µM) pre-treatment (started 1 h before CGOD and continued during 1 h of CGOD) protected the cells, being the number of apoptotic cells decreased to 11%. In neuroblastoma (SH-SY5Y) cells, the percentage of apoptotic cells in basal condition was about 8%; this number increased up to 18% as a consequence of cell exposure to 1 h CGOD. The pretreatment with guanosine (in the same conditions as described above) significantly decreased the proportion of apoptotic cells, to 12%.

**DISCUSSION**

Extracellular guanosine has a variety of trophic effects. It stimulates proliferation of astrocytes (Kim et al., 1991 and Ciccarelli et al., 2000) and other cells (Rathbone et al., 1992), stimulates the synthesis and release by astrocytes and microglia of neurotrophic and pleiotrophic factors such as NGF, S-100ß and TGF-ß (Middlemiss et al., 1995; Caciagli et al., 2000; Ciccarelli et al., 1999; Di Iorio et al., 2001), exerts possible neurotrophic effects (Frizzo et al., 2001). Guanosine also enhances outgrowth of neuritic processes from neurons and neuron-like cells (Gysbers and Rathbone, 1992: Rathbone and Juurlink, 1993) and does not induce apoptosis in astrocytes (Di Iorio et al., 2001).

As the inventors have reported above, certain intracellular pathways are involved in the transduction signal of apoptosis. The PI 3-kinase/ Akt pathway is important in delivering an anti-apoptotic signal and the p38 mitogen-activated protein kinase pathway for promoting apoptosis. The inventors have demonstrated, that extracellular guanosine, alone, does not induce apoptosis (Di Iorio et al., 2002), however, when the PI 3-kinase pathway is inhibited (LY294002), gaunosine does induce apoptosis in cultured rat astrocytes. To verify that extracellular guanosine does work through this

particular pathway, the inventors have confirmed an increase in the phosphorylation of Akt by Western blot analysis and the production of the anti-apoptotic protein Bcl-2 by RT-PCR. In order to give credence to the hypothesis that extracellular guanosine is capable of rescuing/ protecting cells from an apoptotic stimuli, the inventors pre-treated astrocytes with 300 μM guanosine before the exposure of a known potent inducer of apoptosis, staurosporine, and found that this pre-treatment rescued 50% of the cell population from undergoing apoptosis. Finally the anti-apoptotic effect of guanosine was abolished by cell pre-treatment with LY294002.

To determine if the anti-apoptotic effect of guanosine was a phenomenon occurring only in astrocytes, the inventors examined the antiapoptotic effect of extracellular guanosine on differentiated SK-N-SH neuronal-type cells. The inventors found that once again, guanosine does not induce apoptosis in these types of cells. The inventors administered a different inducer of apoptosis to correlate the anti-apoptotic effect of guanosine directly to Alzheimer's disease, where neurons are continually being lost due to apoptosis due to the accumulation of senile plaques caused by abnormal processing of amyloid precursor protein. In these cultures the inventors found that extracellular guanosine was able to once again rescue 50% of the cells from the β-amyloid apoptotic stimulus.

To determine whether this effect was limited to the non-adenine based purine nucleoside, guanosine, the inventors began testing other purine nucleosides such as inosine. The inventors found that inosine, like guanosine does not induce apoptosis in cultured rat astrocytes (Di Iorio et al., 2002) and has neurorestorative effects (Benowitz et al., 1998, 1999). The inventors found that this nucleoside is also capable of protecting neuronal-type cells by more than 50%, from undergoing apoptosis.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

**Table 1**

Effect of guanosine (A. 100 µM or B. 300 µM) alone or in combination on the percent of β-amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 24 hours.

A.

| Treatment | Average |
|---|---|
| Control | 6 ± 1.5 |
| DMSO vehicle | 10 ± 3.5 |
| NaOH vehicle | 10 ± 5.7 |
| Staurosporine 10 nM | 32 ± 12 |
| Guanosine 100 µM | 4 ± 1.5 |
| β-amyloid 5 µM | 21 ± 7.8 |
| Guanosine 100 µM + β-amyloid 5 µM | 13 ± 6.4 |
| β-amyloid 10 µM | 28 ± 8.9 |
| Guanosine 100µM + β-amyloid 10 µM | 6 ± 1.5 |

B.

| Treatment | Average |
|---|---|
| Control | 5.5 ±2.1 |
| DMSO vehicle | 11.5 ± 4.9 |
| NaOH vehicle | 5 ±1.4 |
| Staurosporine 10 nM | 42 ± 2.8 |
| Guanosine 300 µM | 7 ± 1.4 |
| β-amyloid 5 µM | 16.5 ± 0.7 |
| Guanosine 300 µM + β-amyloid 5 µM | 8.5 ± 3.5 |
| β-amyloid 10 µM | 25.5 ± 9.2 |
| Guanosine 300µM + β-amyloid 10 µM | 11.5 ± 3.5 |

Cells were pre-treated with guanosine (100 µM or 300 µM) for 1 hour before exposure to β-amyloid. Cells were then exposed to (25-35) β-amyloid (5 µM or 10 µM) for the duration of the experiment. Exposure to guanosine and / or β-amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point by staining with acridine orange/ ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total number of cells counted. Each average represents the mean ± SD of (A) 3 independent experiments or (B) 2 independent experiments.

**Table 2**

Effect of guanosine (A. 100 µM or B. 300 µM) alone or in combination on the percent of β-amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 48 hours of treatment.

A.

| Treatment | Average |
|---|---|
| Control | 4.7 ± 1.2 |
| DMSO vehicle | 12 ± 6.1 |
| NaOH vehicle | 11.3 ± 7.6 |
| Staurosporine 10 nM | 41.3 ± 16.2 |
| Guanosine 100 µM | 7 ± 3.6 |
| β-amyloid 5 µM | 28.7 ± 8.1 |
| Guanosine 100 µM + β-amyloid 5 µM | 12 ± 5 |
| β-amyloid 10 µM | 36.7 ± 12.1 |
| Guanosine 100µM + β-amyloid 10 µM | 15.3 ± 6.4 |

B.

| Treatment | Average |
|---|---|
| Control | 3.5 ± 2.1 |
| DMSO vehicle | 10.5 ± 0.7 |
| NaOH vehicle | 16 ± 2.8 |
| Staurosporine 10 nM | 42 ± 8.5 |
| Guanosine 300 µM | 12.5 ± 3.5 |
| β-amyloid 5 µM | 33.5 ± 0.7 |
| Guanosine 300 µM + β-amyloid 5 µM | 11 ± 1.4 |
| β-amyloid 10 µM | 48 ± 9.9 |
| Guanosine 300µM + β-amyloid 10 µM | 18 ± 2.8 |

Cells were pre-treated with guanosine (100 µM or 300 µM) for 1 hour before exposure to β-amyloid. Cells were then exposed to (25-35) β-amyloid (5 µM or 10 µM) for the duration of the experiment. Exposure to guanosine and/or β-amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point by staining with acridine orange/ ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total number of cells counted. Each average represents the mean ± SD of (A) 3 independent experiments or (B) 2 independent experiments.

**Table 3**

Effect of inosine 100 µM alone or in combination on the percent of ß-amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 24 (A) or 48 hours (B) following treatment.

A.

| Treatment | Average |
|---|---|
| Control | 9 |
| DMSO vehicle | 8 |
| Staurosporine 10 nM | 26 |
| Inosine 100 µM | 6 |
| β-amyloid 5 µM | 25 |
| Inosine 100 µM + β-amyloid 5 µM | 11 |
| β-amyloid 10 µM | 25 |
| Inosine 100µM + β-amyloid 10 µM | 17 |

B.

| Treatment | Average |
|---|---|
| Control | 7 |
| DMSO vehicle | 6 |
| Staurosporine 10 nM | 32.5 |
| Inosine 100 µM | 3 |
| β-amyloid 5 µM | 25 |
| Inosine 100 µM + β-amyloid 5 µM | 10.5 |
| β-amyloid 10 µM | 20.5 |
| Inosine 100 µM + β-amyloid 10 µM | 12.5 |

Cells were pre-treated with inosine (100 µM ) for 1 hour before exposure to β-amyloid. Cells were then exposed to (25-35) β-amyloid (5 µM or 10 µM) for the duration of the experiment. Exposure to inosine and/or β-amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point (A) of 48-hour time point (B) by staining with acridine orange/ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total cell number counted.

**Table 4.** Effect of selected inhibitors of intracellular pathways on apoptosis induced by staurosporine in rat brain cultured astrocytes in the absence and in the presence of guanosine

| | No Guano sine | | Guanosine 300 µM | |
|---|---|---|---|---|
| | **Basal** | **Staurosporine 100 nM** | **Basal** | **Staurosporine 100 nM** |
| Control | 5.4±0.7 (n = 5) | 53.6±4.9 (n = 6) § | 1.9±0.5 (n = 6) | 23.0±3.1 (n = 6) # |
| **SB202190 1 µM** | 4.9±1.1 (n = 5) | 17.1±2.6 (n = 5) *** | 2.8±0.4 (n = 5) | 16.3±1.7 (n = 5) * |
| **z-VAD-fmk 200 µM** | 4.4±0.8 (n = 5) | 13.2±1.9 (n = 5) *** | 2.4±0.2 (n = 5) | 15.2±1.6 (n = 5) * |
| **SB202190+z-VAD-fmk** | 4.1±0.5 (n = 5) | 7.7±1.7 (n = 5) *** | 2.3±0.5 (n = 5) | 10.4±2.2 (n = 5) ** |
| **LY294002 30 µM** | 6.1±1.2 (n = 6) | 59.2±5.9 (n = 6) | 7.1±1.3 (n = 6) ** | 57.9±6.8 (n = 6) |

Cells were exposed for 3 h to staurosporine (100 nM) in a DMEM with 2% FCS to induce apoptosis (positive control). Guanosine (300 µM) was added to the culture medium 1 h before staurosporine exposure. When indicated, cell pretreatments with [4-(4-(fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI] (SB202190) or [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002) or z-VAD-fmk were carried out starting from 30 min before astrocyte exposure to guanosine and/or staurosporine and maintained for the entire duration of the experiment. Astrocyte apoptosis was analysed at the 24 h time point by acridine orange/ethidium brominde staining as reported in the method section. Apoptotic cells were counted as a percentage of the total cell number. Values are mean ± SEM of 5-6 independent experiments. Statistical significance was evaluated by Student's t-test; § $P < 0.001$ compared with the respective basal value; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared with respective positive control; # $p < 0.001$ compared with positive control in the absence of guanosine.

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